Immunity, Vol. 5, 417–428, November, 1996, Copyright ©1996 by Cell Press

Fyn Can Partially Substitute for Lck in T Lymphocyte Development

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Summary

Lck, a Src family tyrosine kinase, transduces signals important for the development of αβ and γδ T cells. However, T cell development is only partially compromised in Lck-deficient mice, suggesting that other kinases may also transduce pre-TCR or TCR signals. One candidate is Fyn, a Src kinase coexpressed with Lck in immature and mature T cells. Here we show that T cell development is completely compromised in *lck*$^{-/-}$*fyn*$^{-/-}$ mice. In addition, we demonstrate that expression of a gain-of-function mutant *fyn(T)* transgene completely restores production of immature CD4/CD8 double positive thymocytes and γδ T cells and improves the representation of CD4 or CD8 single positive thymocytes. These observations reveal that Fyn can subserve some Lck-like functions in T cell development.

Introduction

Two structurally distinct T cell receptor β (TCRβ)-containing receptor complexes regulate T lymphocyte development. The pre-TCR complex, thought to consist of pre-Tα, TCRβ, and CD3 proteins, is expressed on CD25$^+$ CD4/CD8 double negative (DN) thymocytes (reviewed by Levelt and Eichmann, 1995). In mutant mice lacking recombinase-activating genes 1 or 2 (*RAG1* or *RAG2*) (Mombaerts et al., 1992b; Shinkai et al., 1992), TCRβ (Mombaerts et al., 1992a), pre-Tα (Fehling et al., 1995), CD3ε (Malissen et al., 1995), or TCRζ (Liu et al., 1993; Love et al., 1993; Malissen et al., 1993; Ohno et al., 1993), development of double positive (DP) thymocytes from CD25$^+$ DN progenitors is diminished or completely abrogated, reducing thymic cellularity 10- to 100-fold. Thus, expression of the pre-TCR complex regulates a critical checkpoint in thymocyte development. Consistent with this notion, productively rearranged TCRβ transgenes (Kishi et al., 1991; Mombaerts et al., 1992b; Shinkai et al., 1993; Shores et al., 1993) or anti-CD3ε treatment (Levelt et al., 1993; Jacobs et al., 1994; Shinkai and Alt, 1994) restores thymic cellularity and the development of DP thymocytes in severe combined immunodeficient (*scid*) or RAG1- or RAG2-deficient mice. Collectively, these results demonstrate that pre-TCR signals stimulate TCRβ$^+$ DN progenitors to proliferate, down-regulate CD25, and express the CD4 and CD8 major histocompatibility complex (MHC) coreceptors. At this stage, TCRα expression begins, and TCRαβ-mediated recognition of peptide–MHC ligands expressed on thymic stromal cells selects some DP thymocytes to mature into CD4$^+$ helper or CD8$^+$ cytotoxic T cells (reviewed by Guidos, 1996).

Fyn and Lck, two lymphocyte-restricted members of the Src tyrosine kinase family, have both been shown to play important roles in TCRαβ/CD3-mediated signal transduction in mature T cells (reviewed by Anderson et al., 1994; Weiss and Littman, 1994). However, Fyn plays no essential role in pre-TCR- or TCRαβ-induced maturation, since normal numbers of DP and single positive (SP) thymocytes are generated in *fyn*$^{-/-}$ mice (Appleby et al., 1992; Stein et al., 1992; Swan et al., 1995) and expression of a dominant-negative *fyn* allele does not impair T cell development (Cooke et al., 1991). In contrast, multiple lines of evidence point to an important role for Lck at both stages of T cell development. First, the ability of TCRβ transgenes or anti-CD3ε injection to restore normal DP thymocyte development to *RAG1*$^{-/-}$ or *RAG2*$^{-/-}$ mice requires Lck activity (Mombaerts et al., 1994; Levelt et al., 1995; Wu et al., 1996). Second, overexpression of a constitutively active *lck* transgene promotes the development of DP thymocytes in the absence of TCRβ expression (Mombaerts et al., 1994), suggesting that Lck functions downstream of the pre-TCR complex in regulating the DN to DP transition. In addition, Lck signals regulate allelic exclusion of TCRβ gene rearrangement in DN thymocytes (Anderson et al., 1992, 1993). Lck has also been implicated in TCRαβ-mediated signaling (McCarthy et al., 1988; Nakayama et al., 1989, 1990, 1991; Wiest et al., 1993, 1996; van Oers et al., 1996), positive selection (Teh et al., 1991; Carrera et al., 1992a, 1992b; van Oers et al., 1992; Hashimoto et al., 1996), and CD4/CD8 lineage commitment (Itano et al., 1996) of DP thymocytes. Finally, Lck is required for the development of some subsets of γδ T cells (Penninger et al., 1993; Kawai et al., 1995).

Although these observations define an important role for Lck-mediated signaling events during development of γδ and αβ T cells, it is not clear whether Lck is essential for these processes. Analyses of mice overexpressing a dominant-negative *lck* transgene suggest an obligate role for Lck in pre-TCR signaling, since development of DP thymocytes and allelic exclusion of TCRβ are both completely abrogated in these mice (Anderson et al., 1993; Levin et al., 1993). By contrast, some accumulation of DP thymocytes does occur in Lck-deficient mice created by targeted disruption of *lck* (Molina et al., 1992).

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Moreover, *lck*$^{-/-}$ mice exhibit efficient allelic exclusion of TCRβ (Wallace et al., 1995). Similarly, *RAG1*$^{-/-}$*lck*$^{-/-}$ mice still develop small numbers of DP thymocytes in response to expression of a TCRβ transgene (Mombaerts et al., 1994) or anti-CD3ε treatment (Levelt et al., 1995; Wu et al., 1996). Finally, small numbers of peripheral SP T cells develop in *lck*$^{-/-}$ mice (Molina et al., 1992), arguing that, in the absence of Lck, some other signaling molecule can subserve its functions in positive selection of DP thymocytes into the SP lineages.

One obvious candidate for such a signaling element is Fyn, since it is expressed at comparable levels to Lck in DN and DP thymocytes (Olszowy et al., 1995) and is known to associate with CD3 proteins (Gauen et al., 1992). Although Fyn does not play an essential role in T cell development, we hypothesized that Fyn might play a redundant role in pre-TCR or TCRαβ/CD3 signaling (or both). To address this possibility, we generated *lck*$^{-/-}$ mice harboring either recessive loss-of-function or dominant gain-of-function *fyn* alleles. Thymic cellularity in *lck*$^{-/-}$*fyn*$^{-/-}$ mice was reduced 5- to 10-fold relative to *lck*$^{-/-}$ mice, and DP thymocytes, peripheral TCRαβ T cells, and TCRγδ T cells were virtually undetectable. These data indicate that Fyn mediates development of DP thymocytes in *lck*$^{-/-}$ mice. This notion was further supported by our finding that a constitutively active mutant *fyn(T)* transgene (*TFF*) could completely restore DP thymocyte development in *lck*$^{-/-}$ mice. However, in contrast with the analogous gain-of-function *lck* transgene (Mombaerts et al., 1994), the *TFF* transgene could not promote efficient generation of DP thymocytes in *RAG1*$^{-/-}$ mice. Finally, we provide evidence that Lck is important for signal transduction during the DP to SP transition and that this Lck function can be partially subserved by the *fyn TFF* transgene.

Results

Profound Failure of T Cell Development in *lck*$^{-/-}$*fyn*$^{-/-}$ Mice

To determine whether the small numbers of DP thymocytes found in Lck-deficient mice develop in a Fyn-dependent manner, we bred *lck*$^{-/-}$*fyn*$^{-/-}$ and *lck*$^{-/-}$*fyn*$^{+/-}$ mice. Consistent with previous reports (Molina et al., 1992), flow cytometric analysis of lymph node cells from 3- to 6-week-old *lck*$^{-/-}$ mice demonstrated a 10- to 20-fold reduction in the frequency and absolute number of CD4$^+$ and CD8$^+$ TCRαβ$^+$ T cells (Figure 1A; Table 1). In contrast, TCRαβ$^+$ T cells were reduced by less than 2-fold in Fyn-deficient mice (Table 1). The number of lymph node TCRγδ$^+$CD3ε$^+$ cells was similar in Lck-deficient and Fyn-deficient mice and was reduced 2-fold or less relative to normal B6 mice. However, TCRαβ$^+$ or TCRγδ$^+$ cells were virtually undetectable in the lymph nodes (Figure 1A; Table 1) or spleen (data not shown) of the double mutant mice, demonstrating that development of both T cell lineages requires the participation of either Lck or Fyn. In contrast, normal numbers of B cells were present in single and double mutant mice (data not shown).

Analysis of thymocyte number and phenotype suggested that the lack of peripheral TCRαβ$^+$ cells in mice deficient for both Lck and Fyn derived from a profound and early block in thymocyte maturation. In *lck*$^{-/-}$*fyn*$^{-/-}$ mice, thymic cellularity was 1%–5% of normal and thymocytes were predominantly large DN cells, with only 1%–3% progressing to the DP stage (Figure 1B). By contrast, thymic cellularity in *lck*$^{-/-}$*fyn*$^{+/+}$ mice was 10%–20% normal, and 40%–60% of the thymocytes were small DP cells (Molina et al., 1992; Figure 1B). Thymocyte number and phenotype were similar in *lck*$^{-/-}$*fyn*$^{+/+}$ and *lck*$^{-/-}$*fyn*$^{+/-}$ mice, indicating that *fyn* gene dosage had no effect on thymocyte development in the absence of Lck. The severe paucity of DP thymocytes in the double mutant mice was accompanied by an accumulation of immature CD44$^{-/lo}$CD25$^+$ thymocytes, at the expense of their CD44$^-$CD25$^-$ progeny (Figure 1B). Thus, abrogation of Lck and Fyn expression mediates a developmental arrest virtually identical to that in pre-TCR-deficient *RAG1/2*$^{-/-}$ and *scid* mice or in mice expressing a high level of catalytically inactive Lck (reviewed by Anderson and Perlmutter, 1995; Levelt and Eichmann, 1995).

Low levels of TCRβ (Figure 1B) and CD3ε (data not shown) were detected on the surface of 15%–25% of *lck*$^{-/-}$*fyn*$^{-/-}$ thymocytes, arguing that developmental arrest was not due to failure to express a TCRβ/CD3ε-containing pre-TCR complex. TCRβ$^+$ thymocytes in the double mutant mice were predominantly CD25$^+$ and HSA^{hi}, whereas TCRβ$^+$ thymocytes in B6 mice were virtually all CD25$^-$ (Figure 1B; data not shown), suggesting that loss of CD25 from pre-TCR$^+$ thymocytes was substantially impaired in the absence of Lck and Fyn. CD25$^-$TCRβ$^+$ thymocytes developed in *lck*$^{-/-}$*fyn*$^{+/-}$ mice, but this process appeared to be inefficient or slower than normal, since up to 20% of TCRβ$^+$ thymocytes were CD25$^+$ (Figure 1B). Collectively, these results suggest that pre-TCR signaling, which regulates the loss of CD25, acquisition of CD4 and CD8, and clonal expansion of TCRβ$^+$ thymocytes, is significantly impaired in the absence of Lck, but is completely compromised in the absence of both Lck and Fyn. Thus, Fyn can partially compensate for the absence of Lck in the pre-TCR-mediated DN to DP transition.

Expression of a Gain-of-Function *fyn* Transgene Restores DP Thymocyte Development in Lck-Deficient Mice

Src family tyrosine kinases contain a tyrosine residue near the C-terminus that negatively regulates catalytic activity. Phosphorylation of Tyr-528 negatively regulates Fyn kinase function, and substitution of this residue with phenylalanine generates a more active kinase (Davidson et al., 1992). Expression of the analogous gain-of-function *lck* transgene directs the maturation of DP thymocytes in the absence of TCRβ rearrangement (Anderson et al., 1992; Mombaerts et al., 1994), and this effect is not dependent on overexpression of mutant relative to endogenous Lck. Thus, Lck functions genetically downstream of the pre-TCR complex to promote the DN to DP transition. These observations prompted us to examine whether a gain-of-function *fyn* transgene could function similarly in *RAG1*$^{-/-}$ mice and whether it could restore

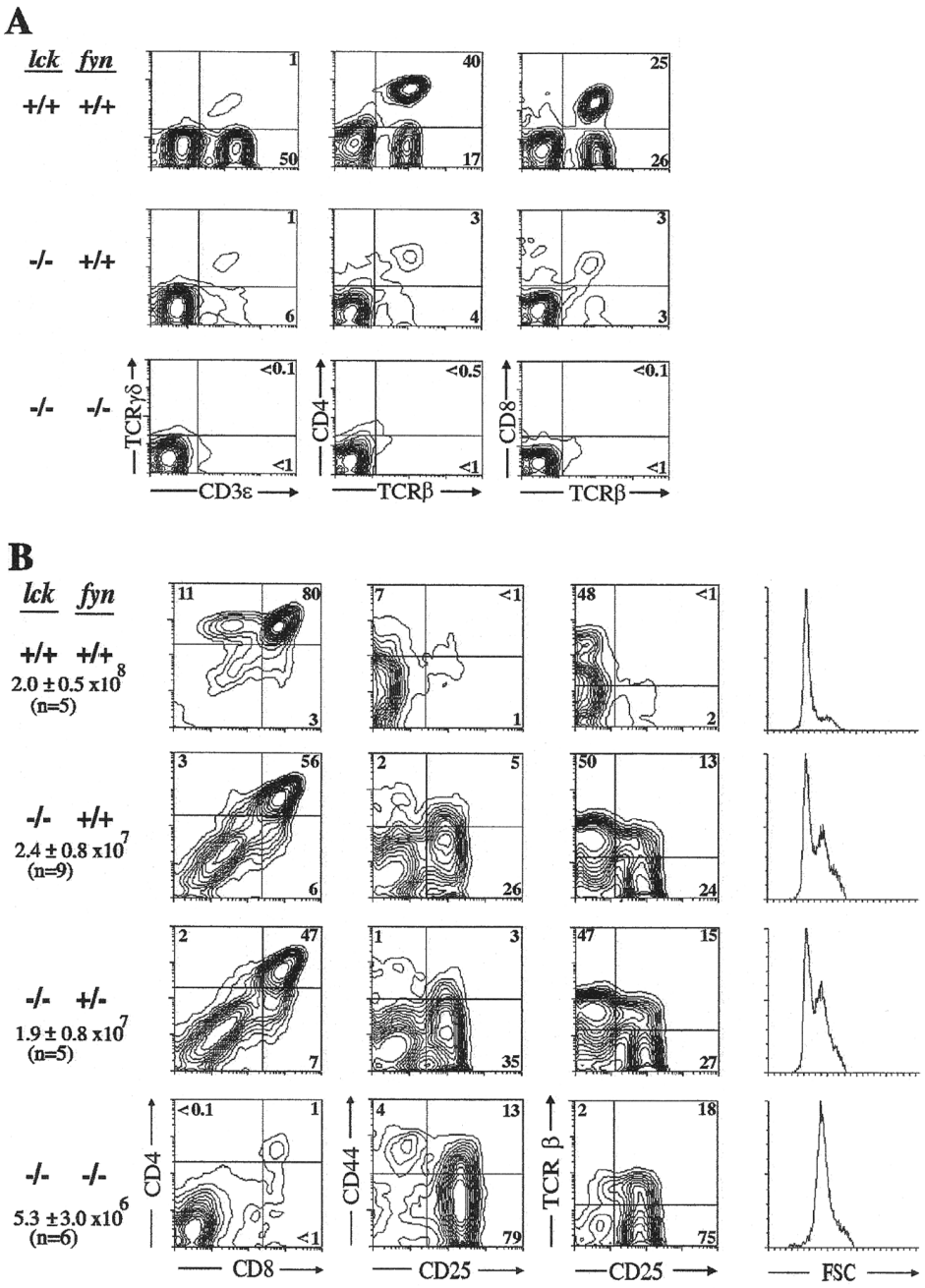

Figure 1. Abrogation of T Cell Development in $lck^{-/-}fyn^{-/-}$ Mice

(A) Absence of αβ and γδ T cells in the periphery of $lck^{-/-}fyn^{-/-}$ mice. The plots display two-color analyses of CD3ε versus TCRγδ, TCRβ versus CD4, and TCRβ versus CD8 expression in lymph node cells from 3- to 6-week-old mice of each genotype. Numbers on each plot indicate the percentage of cells in that quadrant. Quadrant markers were set based on staining with isotype-matched control antibodies. Frequencies of <0.5% were considered negative based on comparison with isotype-matched control stains.

(B) Early block in thymocyte development in $lck^{-/-}fyn^{-/-}$ mice. Thymocytes from 3- to 6-week-old mice of each genotype were stained with the indicated pairs of antibodies and analyzed by two-color flow cytometry. The numbers below each genotype designation refer to the average number of thymocytes (± SD) for that group. Numbers on each plot indicate the percentage of cells in that quadrant. Quadrant markers were set based on staining with isotype-matched control antibodies, except for CD4 versus CD8 and CD44 versus CD25 stains, in which the quadrants demarcate the prominent DP or $CD25^{+}CD44^{lo}$ subsets, respectively.

normal thymocyte development in $lck^{-/-}$ mice. A murine *fyn(T)* cDNA, referred to as *TFF*, was engineered to harbor the tyrosine to phenylalanine gain-of-function mutation at position 528 and was cloned into the p1017 transgene expression cassette, which contains the transcriptional control elements of the *lck* proximal promoter

Table 1. Lymph Node T Cell Subsets in Mice Lacking Lck, Fyn, or Both

Genotype			Number of T Cells	
lck	*fyn*	n	TCRαβ ($\times 10^{-6}$)	TCRγδ ($\times 10^{-5}$)
+/+	+/+	3	14.5 ± 3.5	2.6 ± 0.3
+/+	−/−	4	8.9 ± 3.6	1.7 ± 0.7
−/−	+/+	3	0.9 ± 0.1	1.6 ± 0.8
−/−	−/−	3	ND	ND

n, number of individuals per group; ND, not detectable. Peripheral lymph node cells from individual mice of each genotype were counted and stained with antibodies specific for TCRαβ (H57) or TCRγδ (GL3). The frequency of each subset was multiplied by the total cell count to determine the absolute number present in each animal. Values shown represent the mean ± SD for the number of individual mice analyzed in each group.

(Cooke et al., 1991). A B6 *TFF* transgenic line was established and then crossed to *RAG1*$^{-/-}$*lck*$^{-/-}$ mice (Wu et al., 1996) to generate *TFF*-expressing mice that lacked either *lck* or *RAG1* gene function.

We first evaluated expression of Fyn protein in *TFF* transgenic thymocytes. Transcription from the *lck* proximal promoter is down-regulated in SP relative to DP thymocytes (Reynolds et al., 1990; Wildin et al., 1991), so the *TFF* transgene is expressed primarily in DN and DP thymocytes (data not shown), consistent with the expression patterns of several other transgenes expressed under control of the proximal *lck* regulatory elements (reviewed by Anderson et al., 1994). We therefore compared Fyn protein levels in *TFF* transgenic thymocytes with those in Lck-deficient thymocytes (a mixture of DN and DP cells) and MHC I$^{-/-}$MHC II$^{-/-}$ (primarily DP) thymocytes. Fyn protein was expressed at 2- to 4-fold higher levels in *TFF* transgenic strains relative to these nontransgenic strains (Figure 2; data not shown). Because the signal in *TFF* thymocytes derives from both transgenic and endogenous Fyn, these results suggest that mutant Fyn protein is expressed at 1–2 times that of endogenous wild-type Fyn. Figure 3A shows that the *TFF* transgene can promote the development of a few (<5%) DP thymocytes in *RAG1*$^{-/-}$*lck*$^{+/-}$ mice, but thymic cellularity was not improved. Thus, in contrast with constitutively active Lck, constitutively active Fyn does not efficiently promote the DN to DP transition in the absence of a TCRβ-containing pre-TCR complex.

In contrast with the marginal effect of the *TFF* transgene on thymocyte development in RAG1-deficient mice, the data in Figure 3B demonstrate that it has a profound effect in *RAG1*$^{+/-}$*lck*$^{-/-}$ mice. In *lck*$^{-/-}$ mice, thymic cellularity is 5%–10% of normal owing to a 10- to 20-fold reduction in the production of DP thymocytes (Molina et al., 1992; Wallace et al., 1995), but the *TFF* transgene restored thymic cellularity and development of normal numbers of DP thymocytes to *lck*$^{-/-}$ mice (Figure 3B). As noted previously (Molina et al., 1992), we found that TCRβ/CD3ε expression on *lck*$^{-/-}$ DP thymocytes was 3- to 4-fold higher than on wild-type DP cells (Figure 4A), although a prominent TCR$^{med/hi}$ subset was absent (Figure 3B). We also noted that expression of CD5, which is normally up-regulated in parallel with TCR during thymocyte development (Bendelac et al., 1992; Guidos and Weissman, 1993), was 5- to 6-fold lower on *lck*$^{-/-}$ relative to normal DP thymocytes (Figure 4A). Interestingly, the *TFF* transgene also restored normal levels of thymocyte TCR and CD5 expression to DP thymocytes in *RAG1*$^{+/-}$*lck*$^{-/-}$ mice (Figure 4B). Thus, the *TFF* transgene can replace Lck function to promote the expansion and phenotypic development of DP thymocytes.

The *TFF* Transgene Improves the DP to SP Transition in Lck-Deficient Thymocytes

Lck deficiency also reduces, but does not abrogate, the development of CD4 and CD8 SP T cells (Molina et al., 1992). This reduction could reflect the reduced pool size of DP precursors in *lck*$^{-/-}$ mice or a specific defect in positive selection of Lck-deficient DP thymocytes (or both). In the former case, DP and SP thymocytes should both be reduced in number but present at normal frequencies, as has been shown for interleukin-7-deficient mice (von Freeden-Jeffry et al., 1995). However, very few SP thymocytes are detectable in *lck*$^{-/-}$ mice (Molina et al., 1992), and most thymocytes falling into the SP gates have CD4^{+}CD8^{lo} or CD4^{lo}CD8^{+} transitional phenotypes (Figure 3B). Furthermore, few transitional/CD4 SP thymocytes in *lck*$^{-/-}$ mice expressed mature levels of TCR, CD5, and CD69 (Figure 4A), suggesting that positive selection of DP thymocytes into the CD4 lineage is defective.

Expression of the *TFF* transgene enhanced the production of transitional/SP and TCR$^{med/hi}$ thymocytes in Lck-deficient mice (Figure 3B), suggesting that activated Fyn significantly improves the DP to SP transition in the absence of Lck. This notion was further supported by the observation that transitional/SP thymocytes from *TFF*$^{+}$ *RAG1*$^{+/-}$*lck*$^{-/-}$ mice expressed nearly normal levels of

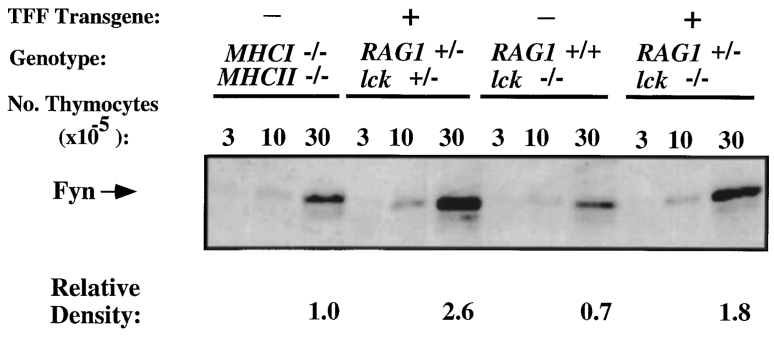

Figure 2. Western Blot Analysis of Fyn Protein Levels in Thymocytes

Postnuclear supernatants from the indicated number of cells were separated by SDS–polyacrylamide gel electrophoresis (nonreducing), transferred to nitrocellulose, and blotted with Fyn-specific antisera. Thymocytes from MHC I$^{-/-}$MHC II$^{-/-}$ mice are primarily DP cells, and Lck-deficient thymocytes consist of approximately equal numbers of DN and DP cells. Densitometric analysis was performed, and the results were normalized to the signal from MHC-deficient thymocytes, which was arbitrarily designated as 1.0.

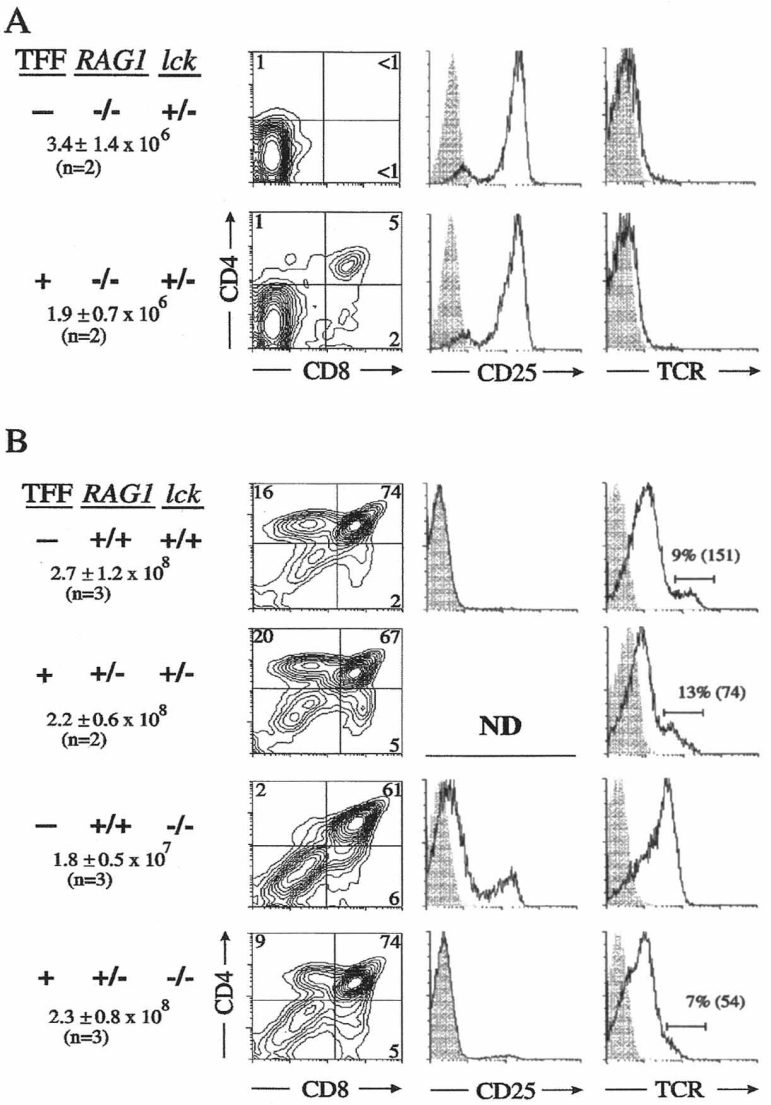

Figure 3. Effect of the *TFF* Transgene on T Cell Development in *RAG1*$^{-/-}$ and *lck*$^{-/-}$ Mice

Thymocytes from 3- to 6-week-old *TFF*$^{+}$ and *TFF*$^{-}$*RAG1*$^{-/-}$ (A) or *RAG1*$^{+/+}$ or *RAG1*$^{+/-}$ mice (B) were counted and analyzed for expression of CD4 versus CD8, CD25, and TCRβ as described for Figure 1B. CD25 expression was not determined (ND) on *TFF*$^{+}$*RAG1*$^{+/-}$*lck*$^{+/-}$ thymocytes. Since all *TFF* transgenic mice were derived from *TFF*$^{+}$ × *TFF*$^{-}$ matings, they express only one copy of the transgene. The histogram markers in (B) denote the mature TCR$^{med/hi}$ subset in each strain. Note that this population is not detected in *TFF*$^{-}$*lck*$^{-/-}$ mice. The numbers in brackets refer to mean fluorescence intensity of cells falling into the TCR$^{med/hi}$ gate.

TCR, CD5, and CD69 (Figure 4B), in contrast with those from *lck*$^{-/-}$ mice (Figure 4A). These data suggest that the *TFF* transgene can partially replace Lck function in promoting the DP to SP transition, but several findings suggested that positive selection of Lck-deficient DP thymocytes was not completely normalized by the *TFF* transgene. First, expression of TCR and CD5 on transitional/SP thymocytes from *TFF*$^{+}$ mice, although improved relative to nontransgenic Lck-deficient thymocytes, was still slightly lower than normal (Figure 4B). Furthermore, although *TFF*$^{+}$ Lck-deficient mice had slightly higher numbers of splenic αβ T cells than *TFF*$^{-}$ Lck-deficient mice, wild-type numbers of αβ T cells were not observed (Table 2), and TCR and CD4 levels remained abnormally low (Figure 5). Notably, however, the *TFF* transgene restored normal numbers of splenic γδ T cells to Lck-deficient mice (Table 2). The *TFF* transgene had relatively little impact on thymocyte development in *RAG1*$^{+/-}$*lck*$^{+/-}$ or *RAG1*$^{+/+}$ *lck*$^{+/+}$ mice, although marginally increased numbers of SP thymocytes expressing slightly reduced TCR levels were sometimes observed (see Figure 3B; data not shown). However, peripheral T cells from these mice had a normal phenotype (Figure 5).

TCRαβ/CD3 and CD4 Signaling in *lck*$^{-/-}$ DP Thymocytes

The above results demonstrate that maturation of Lck-deficient DP thymocytes into the SP lineages is defective and that this can be ameliorated by expression of constitutively active Fyn. These observations suggest that Lck deficiency impairs signal transduction events required for efficient positive selection of DP thymocytes in vivo, in accord with a recent study (Hashimoto et al., 1996). Therefore, we assessed TCR- and CD4-mediated signal transduction in normal and Lck-deficient DP thymocytes. As previously noted by several investigators (Gilliland et al., 1991; Turka et al., 1991; Wiest et al., 1996), we found that aggregation of TCR or CD3 on fresh ex vivo DP thymocytes from normal mice produces only marginal increases in protein tyrosine phosphorylation, but this response can be markedly improved by

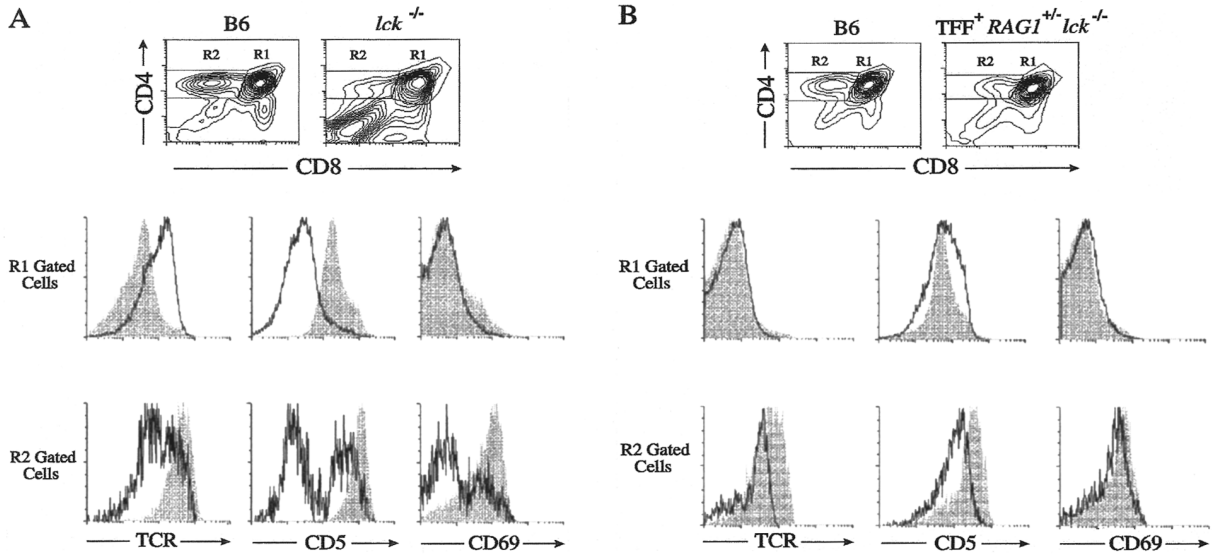

Figure 4. Effect of the *TFF* Transgene on the DP to SP Transition in $lck^{-/-}$ Mice

Thymocytes from 3- to 6-week-old B6 versus $lck^{-/-}$ mice (A) and B6 versus $TFF^{+}lck^{-/-}$ mice (B) were analyzed by three-color flow cytometry for CD4 and CD8 expression versus TCR, CD5, or CD69. For each genotype, the R1 gate defines DP thymocytes and the R2 gate defines transitional/CD4 SP thymocytes. The histogram overlays compare the expression of TCR, CD5, and CD69 on R1- or R2-gated cells from B6 (shaded histograms) versus $lck^{-/-}$ (unshaded histograms) mice (A) or B6 (shaded) versus $TFF^{+}lck^{-/-}$ (unshaded) mice (B).

coaggregation of the TCR with CD4 (Figure 6A). However, CD4-induced tyrosine phosphorylation, most notably of the 120/130 kDa protein substrate, was abrogated by Lck deficiency (Figure 6A). Nonetheless, Lck-deficient DP thymocytes exhibited robust TCR-induced protein tyrosine phosphorylation without CD4 coaggregation, arguing that proximal TCR signaling pathways are intact, and perhaps improved, in the absence of Lck. Distal TCR signaling pathways were also activated, since TCR stimulation increased CD5 and CD69 expression and decreased *RAG1* expression in Lck-deficient DP thymocytes, although these responses were slightly less efficient in the absence of Lck (Figures 6B and 6C). Thus, the TCR can transduce signals leading to activation or maturation of Lck-deficient DP thymocytes in vitro, yet these cells fail to mature normally in vivo.

Discussion

This study has revealed that Fyn and Lck can subserve redundant functions during T cell development, in agreement with the documented ability of Src family kinases to substitute for one another in regulating growth and differentiation of other cell types (Lowell and Soriano, 1996). This redundancy was evident in the development of different T cell lineages ($\alpha\beta$ and $\gamma\delta$), as well as during pre-TCR- and TCR-mediated signaling events required for $\alpha\beta$ T cell development. However, the degree of redundancy was not the same in all of these processes. For example, development of lymph node $\gamma\delta$ T cells was compromised to a similar but minor extent in the absence of Lck or Fyn, but was virtually abolished in the absence of both molecules, suggesting that there is a high degree of functional overlap between these two kinases. In contrast, developmental transitions mediated by the pre-TCR and TCR are severely compromised in the absence of Lck, but remain largely intact in the absence of Fyn, suggesting a greater reliance on Lck in these developmental processes. Nonetheless, we show here that transgenic expression of constitutively active Fyn can almost completely replace Lck function in all of these aspects of T cell development.

Table 2. Effect of *TFF* Transgene on Peripheral T Cell Number in Lck-Deficient Mice

Genotype				Number of T Cells	
lck	*RAG1*	*TFF*	n	$\alpha\beta$ T cells ($\times 10^{-6}$)	$\gamma\delta$ T cells ($\times 10^{-5}$)
+/+	+/+	−	5	47 ± 14	16 ± 3
−/−	+/+	−	5	6 ± 4	4 ± 2
−/−	+/−	+	3	18 ± 5	19 ± 9

n, number of individuals per group. Erythrocyte-depleted spleen cells from individual mice of each genotype were counted, and the absolute number of $\alpha\beta$ and $\gamma\delta$ T cells in each sample was determined as described for Table 1.

Functions of Fyn and Lck in $\gamma\delta$ T Cell Development

We observed small numbers of $TCR\gamma\delta^{+}$ thymocytes in $lck^{-/-}fyn^{-/-}$ animals (data not shown), suggesting that the absence of these cells in spleen and lymph nodes (Figure 1A; Table 1) may reflect defective selection, export from the thymus, peripheral expansion of these cells, or a combination of these factors. Although $V\gamma2^{+}$ T cells are positively selected on MHC class I, development of $V\gamma3^{+}$ skin T cells is class I independent (Wells et al., 1991; Correa et al., 1992). In addition, available evidence suggests that selection of $V\gamma3^{+}$ is not dependent on TCR–ligand interactions (Asarnow et al., 1993). Thus, it remains unclear whether the $\gamma\delta$ TCR or other cell surface receptors interact with Fyn and Lck during $\gamma\delta$ T cell development.

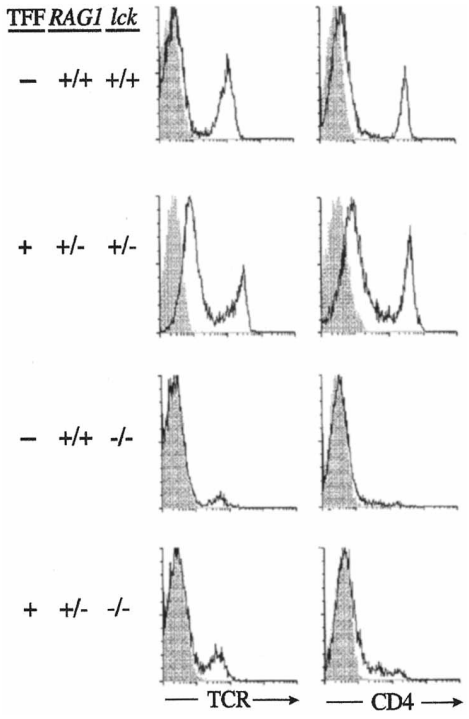

Figure 5. Effect of *TFF* Transgene on the Frequency of TCRβ$^+$ and CD4$^+$ Splenic T Cells

Erythrocyte-depleted spleen cells from individual 3- to 6-week-old mice of each genotype were analyzed for TCRβ and CD4 expression as described for Figure 1. Two to five mice of each genotype were analyzed; a representative animal from each group is shown.

Notably, the *TFF* transgene restored splenic γδ T cell numbers to normal levels in Lck-deficient mice (Table 2), arguing that it can effectively compensate for Lck in this regard. However, the development of Vγ2 transgene-bearing T cells appears to be strictly Lck dependent (Penninger et al., 1993), suggesting that Fyn can replace Lck function for the production of most, but not all, thymically derived γδ T cells. Interestingly, the development of intraepithelial γδ T cells in the gut, which is thought to occur extrathymically (Poussier and Julius, 1995), was previously shown to be Lck independent (Penninger et al., 1993). Studies are in progress to determine whether Fyn plays an essential or a functionally redundant role in the development of this γδ T cell subset.

Functional Redundancy of Lck and Fyn in Pre-TCR-Mediated Positive Selection

Although Fyn plays no essential role in the DN to DP transition (Appleby et al., 1992; Stein et al., 1992), experiments described here show that the development of DP thymocytes in Lck-deficient mice is Fyn dependent (Figure 1). These findings likely explain why *RAG*$^{-/-}$ *lck*$^{-/-}$ mice can still develop small numbers of DP thymocytes after treatment with ionizing radiation or anti-CD3 (Levelt et al., 1995; Wu et al., 1996). In addition, our observations suggest an explanation for the different thymocyte phenotypes of *lck*$^{-/-}$ mice versus mice expressing a dominant-negative mutant *lck* transgene. DP thymocyte development and TCRβ allelic exclusion (Anderson et al., 1993; Levin et al., 1993) are completely abrogated in the latter mice (Anderson et al., 1993; Levin et al., 1993), whereas these events are only partially compromised in *lck*$^{-/-}$ mice (Molina et al., 1992; Wallace et al., 1995). We suggest that the dominant-negative *lck* transgene, which was expressed at 12-fold higher levels than endogenous *lck*, did not permit adventitious signaling by Fyn, which clearly can participate in DP thymocyte development in *lck*$^{-/-}$ mice.

In mice lacking both Lck and Fyn, we observed an accumulation of TCRβ$^+$CD25$^+$ DN thymocytes (Figure 1B), suggesting that pre-TCR-expressing precursors could not transduce signals required for developmental progression and proliferation. In accord with this idea, expression of a constitutively active *fyn* transgene completely obviated the need for Lck function at this stage of T cell development (Figure 3B), suggesting that the two kinases could in principle subserve overlapping functions in the DN to DP transition. However, several observations argue that Fyn and Lck do not function identically in this regard. First, DP thymocyte development is severely impaired in *lck*$^{-/-}$ mice, but is normal in *fyn*$^{-/-}$ mice (Appleby et al., 1992; Molina et al., 1992; Stein et al., 1992). This is not likely due to quantitative differences, since it has been reported that Fyn and Lck are expressed at similar levels in DN and DP thymocytes (Olszowy et al., 1995). Second, transgenic expression of constitutively active Lck both restores normal DP thymocyte development to *RAG1*$^{-/-}$ mice (Mombaerts et al., 1994) and suppresses TCRβ gene rearrangement in wild-type mice (Anderson et al., 1992, 1993), but the analogous *TFF* transgene does not (Figure 3A; data not shown). Again, this difference does not correlate with different levels of transgene expression, since the effect of the mutant Lck was observed when it was expressed at endogenous Lck levels, yet the *TFF* transgene had only marginal effects despite being overexpressed at least 2-fold relative to endogenous Fyn (Figure 2). Finally, overexpression of a dominant-negative *lck* transgene completely abrogates DP thymocyte development, whereas a dominant-negative *fyn* transgene does not affect T cell development (Cooke et al., 1991).

Collectively, these data suggest that Lck and Fyn may interact with an overlapping but not identical set of substrates to mediate pre-TCR signals, either because they possess inherently different substrate specificities or owing to distinct intracellular distributions (Ley et al., 1994). In mature T cells, Fyn and Lck are thought to be activated by aggregation of distinct cell surface receptors, since Fyn is found associated with the cytoplasmic tails of CD3 chains and other surface receptors, whereas at least some cellular Lck is associated, via its unique N-terminal domain, with cysteine residues in the cytoplasmic tails of CD4 and CD8α (Weiss and Littman, 1994). However, once activated, both Fyn and Lck are thought to phosphorylate tyrosine-based activation motifs in TCRζ, CD3ε, CD3γ, and CD3δ and, subsequently, ZAP-70 or Syk (or both), members of a distinct tyrosine kinase family (reviewed by Anderson et al., 1994; Weiss and Littman, 1994). By contrast, neither the intracellular distributions nor the protein substrates of Lck and Fyn in pre-TCR-expressing immature thymocytes have been

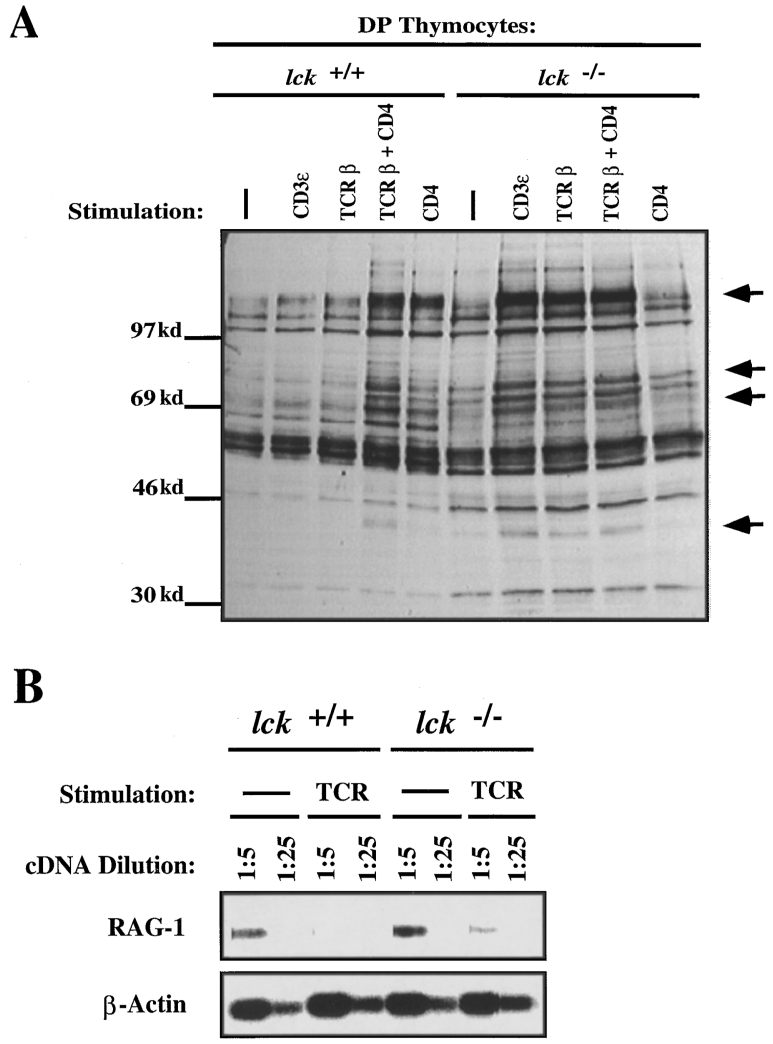

Figure 6. Effect of Lck Deficiency on TCR-Mediated Signal Transduction in DP Thymocytes

(A) TCR/CD4-induced protein tyrosine phosphorylation in $lck^{+/+}$ versus $lck^{-/-}$ DP thymocytes. Purified DP thymocytes from $lck^{+/+}$ and $lck^{-/-}$ mice were cultured for 1 min at 37°C without stimulation or after antibody-mediated cross-linking of the indicated surface molecules. Cellular proteins from equal cell numbers were separated by SDS–polyacrylamide gel electrophoresis (nonreducing), transferred to nitrocellulose, and blotted with a monoclonal anti-phosphotyrosine antibody (4G10). Arrows indicate several proteins that undergo TCR/CD4-inducible tyrosine phosphorylation. Numbers on the left indicate the migration of molecular mass standards.

(B) Reverse transcription–polymerase chain reaction (PCR) analysis of *RAG1* and β-actin transcripts in DP thymocytes cultured overnight alone or with anti-TCRβ. The indicated cDNA dilutions were PCR amplified with primers specific for *RAG1* or β-actin, and the products were fractionated on agarose gels, blotted onto nylon membrane, probed with ^{32}P-labeled *RAG1* or β-actin cDNA fragments, and exposed to a phosphorimager screen. Note that both normal and Lck-deficient DP thymocytes show TCR-induced down-regulation of *RAG1* transcripts, whereas β-actin transcript abundance is similar in all samples.

(C) Basal and TCR-induced CD5 and CD69 expression by normal versus Lck-deficient DP thymocytes. DP thymocytes from each genotype were purified by cell sorting and then cultured overnight alone or with immobilized anti-TCRβ antibody. Histograms show CD5 and CD69 expression on unstimulated (shaded histograms) or anti-TCRβ-stimulated (unshaded histograms) DP thymocytes.

identified. In the simplest model, Fyn and Lck would both be associated with the cytoplasmic tails of pre-TCR components, allowing their direct activation by aggregation of pre-TCR complexes, but this has not yet been demonstrated biochemically and the existence of pre-TCR ligands remains speculative. Moreover, Fyn and Lck also interact with cytokine receptors and other surface molecules, such as CD2, known to be expressed in immature thymocytes (Anderson et al., 1994; Seckinger and Fougereau, 1994; von Freeden-Jeffry et al., 1995). Thus, Lck and Fyn need not be directly associated with the pre-TCR. Substrates phosphorylated by Lck and Fyn in pre-T cells are also unidentified, although ZAP-70 was shown to be activated by CD3 cross-linking of a pre-TCR-expressing immature T cell line (van Oers et al., 1995). However, the absence of ZAP-70 or Syk does not compromise development of DP thymocytes (Cheng et al., 1995; Negishi et al., 1995; Turner et al., 1995). Thus, ZAP-70 and Syk may not be involved in pre-TCR signaling, or they may also play redundant roles in this process.

Role of Lck and Fyn in Positive Selection of DP Thymocytes

Fyn-mediated signal transduction is dispensable for positive selection of DP thymocytes into the CD4 or CD8 lineage (Appleby et al., 1992; Stein et al., 1992; Swan et al., 1995). In contrast, very few SP thymocytes and T cells develop in $lck^{-/-}$ mice (Molina et al., 1992). We

show here that the DP to SP transition is significantly compromised in the absence of Lck, suggesting that Lck-mediated signals are important for positive selection of most DP thymocytes. In accord with this idea, a recent study demonstrated that expression of catalytically inactive Lck specifically in DP cells using the distal Lck promoter effects an unambiguous block in positive selection (Hashimoto et al., 1996).

The role of Lck in positive selection could involve transducing signals from CD4/CD8, the TCR complex, or both. We show here that defective positive selection of Lck-deficient DP thymocytes in vivo does not appear to correlate with defective TCR signaling in vitro (Figure 6). By contrast, we found that CD4-dependent signaling was abrogated in Lck-deficient DP thymocytes (Figure 6A). This defect could impinge on positive selection in vivo, since a recent study (Wiest et al., 1996) showed that activation of ZAP-70, an essential event in positive selection (Negishi et al., 1995), requires coaggregation of CD4 with the TCR in DP thymocytes. However, other defects in Lck-deficient DP thymocytes might also account for their defective maturation into SP T cells in vivo. For example, Lck deficiency caused decreased CD5 and increased TCR expression in DP thymocytes, and both defects were normalized by expression of the *TFF* transgene (Figure 4). Interestingly, these results further suggest that DP thymocytes modulate CD5 levels in response to Lck- and Fyn-dependent signal transduction in vivo. CD5 has been reported to negatively regulate TCR signaling in thymocytes and to play an important role in positive selection (Tarakhovsky et al., 1995), suggesting that abnormal CD5 expression could impair positive selection of Lck-deficient DP thymocytes.

Although endogenous Fyn appears to compensate poorly for Lck in development of transitional/SP thymocytes and peripheral T cells, the *TFF* transgene could partially restore this developmental transition (Figure 4; Table 2), demonstrating that Fyn can transduce signals important for positive selection of DP thymocytes. The differential effectiveness of endogenous wild-type Fyn and transgenic TFF might be explained by the slightly higher expression of the latter in DP thymocytes (Figure 2), but it is important to note that endogenous Fyn expression increases during the DP to SP transition (Cooke et al., 1991), whereas TFF is expected to decrease across this transition owing to its expression under control of the *lck* proximal promoter. Down-regulation of *TFF* expression likely begins during the earliest phases of positive selection, since we have recently shown that overnight TCR engagement of DP thymocytes in vitro significantly decreases transcription from the *lck* proximal promoter (Groves et al., submitted). Positive selection is thought to be a multistep process requiring several TCR engagement events (reviewed by Guidos, 1996), so down-regulation of *TFF* during the late stages of positive selection could account for its failure to restore completely the development of SP T cells in Lck-deficient mice. An equally plausible explanation for the differential effectiveness of endogenous Fyn and transgenic TFF in this regard is that endogenous Fyn may not be activated efficiently by TCR aggregation in vivo, perhaps because positive selection of DP thymocytes usually occurs in response to low affinity TCR–ligand interactions (Jameson et al., 1995). Finally, the tyrosine to phenylalanine substitution at position 528 could alter the substrate specificity of TFF relative to wild-type Fyn.

In summary, our studies have identified overlapping functions of Fyn and Lck at multiple stages of T cell development, further emphasizing the pivotal role that Src family kinases play in this regard.

Experimental Procedures

Mice

All strains were bred and maintained at the Hospital for Sick Children. Mice deficient for either Lck (Molina et al., 1992) or Fyn (Appleby et al., 1992) were intercrossed to generate doubly mutant animals. Peripheral blood from F2 offspring was analyzed by flow cytometry, and Lck-deficient mice were identified based upon a reduction of TCRβ^+ T cells relative to C57BL/6 (B6) mice. The *fyn* genotype of these animals was determined by a PCR-based assay using genomic tail DNA. A sense primer (5′ *fyn*, CAG GTC TCT GCT GCC GCC TAG) from within *fyn* (T) exon 7A and an antisense primer (3′ *fyn*, CGA GTC ACG TGC AAC TTC CCA) from exon 7B amplified a 600 bp fragment of the wild-type *fyn* allele. In a second reaction, the 5′ *fyn* primer and a 3′ *neomycin* primer SDL23 (Appleby et al., 1992) were used to amplify a 1.6 kb fragment of the disrupted *fyn* (T) allele, which contains the *neomycin* gene inserted into exon 7B. The amplification cycle (1 min at 94°C, 90 s at 55°C, and 75 s at 72°C) was repeated 37 times in a Perkin–Elmer 480 thermalcycler. PCR products were resolved on 2% agarose gels containing 0.5 μg/ml ethidium bromide and photographed under UV light.

A B6 *TFF* transgenic line (5525) was established using standard procedures (Cooke et al., 1991) and then crossed to *RAG1*$^{-/-}$*lck*$^{-/-}$ mice (Wu et al., 1996). *TFF*$^+$ F1 progeny were identified by slot-blot screening of tail DNA with a human growth hormone probe as previously described (Cooke et al., 1991) and then backcrossed to *RAG1*$^{-/-}$*lck*$^{-/-}$ mice. Backcross progeny were then typed for the presence of the *TFF* transgene and for their *RAG1* and *lck* genotype as previously described (Wu et al., 1996).

Antibodies and Flow Cytometry

Fluorochrome-conjugated antibodies specific for the following surface molecules were prepared using standard techniques: CD4 (GK1.5), CD8α (53-6.7 or YTS169.4), CD5 (53-7.3), TCRβ (H57-597), CD3ε (YCD3-1 or 145-2C11), and CD44 (IM178). The remaining antibodies were purchased: GL3 (TCRγδ) and 7D4 (CD25) (Pharmingen, San Diego, CA); 4G10 (Upstate Biotechnology, Lake Placid, NY); horseradish peroxidase (HRP)-conjugated protein A and HRP-conjugated goat anti–mouse IgG (Amersham, Arlington Heights, IL). Anti-Fyn polyclonal rabbit antisera were provided by Dr. A. Veillette (McGill University, Montreal, Quebec).

For flow cytometric analyses, single cell suspensions from thymus, lymph nodes, and erythrocyte-depleted peripheral blood and spleen were prepared in HBSS containing 2% calf serum and 10 mM HEPES and stained for surface markers as described previously (Groves et al., 1995; Guidos et al., 1995). All antibodies were used at saturating concentrations. The secondary reagents used included avidin–phycoerythrin (PE) (Caltag, San Francisco, CA) and a tandem conjugate of avidin–Cychrome-5/PE that was prepared by conjugating PE (Molecular Probes, Eugene, OR) to Cychrome-5 (Biological Detection Systems, Pittsburgh, PA) according to the instructions of the manufacturer. Rat IgG2a, rat IgG2b, and hamster IgG isotype control antibodies conjugated to fluorescein, biotin, or PE were purchased from Pharmingen and used at 1–2 μg/ml. Two- or three-color immunofluorescence was analyzed on a FACScan flow cytometer with LYSIS II software (Becton Dickinson, Mountain View, CA) as previously described (Guidos et al., 1990; Fischer et al., 1995). Dead cells and debris were excluded from the analysis on the basis of low forward scatter or high propidium iodide fluorescence (or both).

Purification of DP Thymocytes

DP thymocytes from *lck*$^{+/-}$ and *lck*$^{-/-}$ mice were purified using a dual laser FACSTAR Plus cell sorter (Becton Dickinson). Cells were

first stained with saturating concentrations of fluorescein isothiocyanate–conjugated anti-CD5 (53-7.3) and PE-conjugated anti-CD8 (53-6.7), followed by cell sorting of the $CD5^{lo}CD8^{hi}$ population. The sorted cells were 99% DP thymocytes, as assessed by staining an aliquot of purified cells with saturating concentrations of biotinylated anti-CD4 (GK1.5) followed by avidin–Cychrome-5/PE. This procedure allowed purification of DP thymocytes without engaging either the TCR or CD4 molecules. Studies of TCR-induced changes in CD5, CD69, and *RAG1* expression were carried out as described elsewhere (Fischer et al., 1995; Groves et al., submitted).

Western Blot Analyses

Anti-Fyn immuoblotting was performed on cells lysed at 5×10^7 cells per milliliter with 50 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 20 μM Na_3VO_4, 50 mM NaF, 1% NP-40, 20 μg/ml leupeptin, and 20 μg/ml aprotinin. Insoluble material was removed by microcentrifugation at 16,000 rpm for 15 min at 4°C. Postnuclear supernatants from the indicated numbers of cell equivalents were resolved on a 8% nonreducing SDS–polyacrylamide gel, transferred to nitrocellulose, probed with anti-Fyn serum and HRP-conjugated protein A, and then developed using the enhanced chemiluminescence detection system (Amersham). For studies of TCR-induced protein tyrosine phosphorylation, DP thymocytes purified as described above were stained at 5×10^7 per milliliter with biotinylated YCD3-1, H57, GK1.5, or H57 plus GK1.5 for 20 min at 4°C. After washing, cells were resuspended at 2.5×10^7 per milliliter and prewarmed to 37°C for 3 min. Biotinylated antibodies were then cross-linked by the addition of 10 μg/ml avidin (Molecular Probes) for 1 min at 37°C. The reaction was stopped by addition of 1 ml of cold PBS containing 400 μM Na_3VO_4. Cells were lysed as described above for Fyn immunoblotting, and postnuclear supernatants were resolved on an 8% reducing SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with 4G10, followed by HRP-conjugated anti–mouse IgG.

Acknowledgments

Correspondence should be addressed to C. J. G. We thank G. Knowles for assistance with flow cytometry, Dr. A. Veillette for Fyn-specific antisera, Drs. T. W. Mak and E. Timms for $lck^{-/-}$ mice, Drs. L. Glimcher and M. Grusby for MHC-deficient mice, Dr. G. Wu for $RAG1^{-/-}lck^{-/-}$ mice, and Drs. M. Julius and J. Danska for helpful discussions and reading the manuscript. In addition, we thank Drs. N. van Oers and A. Weiss for sharing data prior to publication. This work was supported by the Medical Research Council, the National Cancer Institute of Canada, the Leukemia Research Fund, and the Hospital for Sick Children RESTRACOM fund. R. M. P. is an Investigator of the Howard Hughes Medical Institute.

Received May 23, 1996; revised August 22, 1996.

References

Anderson, S.J., and Perlmutter, R.M. (1995). A signaling pathway governing early thymocyte maturation. Immunol. Today *16*, 99–105.

Anderson, S.J., Abraham, K.M., Nakayama, T., Singer, A., and Perlmutter, R.M. (1992). Inhibition of T-cell receptor β chain rearrangement by overexpression of the non-receptor protein tyrosine kinase $p56^{lck}$. EMBO J. *11*, 4877–4886.

Anderson, S.J., Levin, S.D., and Perlmutter, R.M. (1993). Protein tyrosine kinase $p56^{lck}$ controls allelic exclusion of T-cell receptor β-chain genes. Nature *365*, 552–554.

Anderson, S.J., Levin, S.D., and Perlmutter, R.M. (1994). Involvement of the protein tyrosine kinase $p56^{lck}$ in T cell signaling and thymocyte development. Adv. Immunol. *56*, 151–178.

Appleby, M.W., Gross, J.A., Cooke, M.P., Levin, S.D., Qian, X., and Perlmutter, R.M. (1992). Defective T cell receptor signaling in mice lacking the thymic isoform of $p59^{fyn}$. Cell *70*, 751–763.

Asarnow, D.M., Cado, D., and Raulet, D.H. (1993). Selection is not required to produce invariant T-cell receptor γ gene junctional sequences. Nature *362*, 158–160.

Bendelac, A., Matzinger, P., Seder, R.A., Paul, W.E., and Schwartz, R.H. (1992). Activation events during thymic selection. J. Exp. Med. *175*, 731–742.

Carrera, A.C., Baker, C., Roberts, T.M., and Pardoll, D.M. (1992a). Tyrosine kinase triggering in thymocytes undergoing positive selection. Eur. J. Immunol. *22*, 2289–2294.

Carrera, A.C., Wee, S.-B., Roberts, T.M., and Pardoll, D.M. (1992b). Selective association between MHC class I-restricted T cell receptors, CD8, and activated tyrosine kinases on thymocytes undergoing positive selection. J. Immunol. *149*, 3142–3149.

Cheng, A.M., Rowley, B., Pao, W., Hayday, A., Bolen, J.B., and Pawson, T. (1995). Syk tyrosine kinase required for mouse viability and B-cell development. Nature *378*, 303–306.

Cooke, M.P., Abraham, K.M., Forbush, K.A., and Perlmutter, R.M. (1991). Regulation of T cell receptor signaling by a *src* family protein-tyrosine kinase ($p59^{fyn}$). Cell *65*, 281–291.

Correa, I., Bix, M., Liao, N.S., Zijlstra, M., Jaenisch, R., and Raulet, D. (1992). Most $\gamma\delta$ T cells develop normally in β2-microglobulin-deficient mice. Proc. Natl. Acad. Sci. USA *89*, 653–657.

Davidson, D., Chow, L.M.L., Fournel, M., and Veillette, A. (1992). Differential regulation of T cell antigen responsiveness by isoforms of the *src*-related tyrosine protein kinase $p59^{fyn}$. J. Exp. Med. *175*, 1483–1492.

Fehling, H.J., Krotkova, A., Saint-Ruf, C., and von Boehmer, H. (1995). Crucial role of the pre-T-cell receptor α gene in development of $\alpha\beta$ but not $\gamma\delta$ T cells. Nature *375*, 795–798.

Fischer, K.-D., Zmuidzinas, A., Gardner, S., Barbacid, M., Bernstein, A., and Guidos, C. (1995). Defective T-cell receptor signaling and positive selection of Vav-deficient $CD4^+CD8^+$ thymocytes. Nature *374*, 474–477.

Gauen, L.K.T., Kong, A.-N.T., Samelson, L.E., and Shaw, A.S. (1992). $p59^{fyn}$ tyrosine kinase associates with multiple T-cell receptor subunits through its unique amino-terminal domain. Mol. Cell. Biol. *12*, 5438–5446.

Gilliland, L.K., Teh, H.-S., Uckun, F.M., Norris, N.A., Teh, S.-J., Schieven, G.L., and Ledbetter, J.A. (1991). CD4 and CD8 are positive regulators of T cell receptor signal transduction in early T cell differentiation. J. Immunol. *146*, 1759–1765.

Groves, T., Katis, P., Madden, Z., Manickam, K., Ramsden, D., Wu, G., and Guidos, C.J. (1995). *In vitro* maturation of clonal $CD4^+CD8^+$ cell lines in response to TCR engagement. J. Immunol. *154*, 5011–5022.

Guidos, C.J. (1996). Positive selection of $CD4^+$ and $CD8^+$ T cells. Curr. Opin. Immunol. *8*, 225–232.

Guidos, C.J., and Weissman, I.L. (1993). Sequential occurrence of positive and negative selection during T lymphocyte maturation. In Molecular Mechanisms of Immunological Self-Recognition, F. Alt and H.J. Vogel, eds. (San Diego, CA: Academic Press), pp. 137–147.

Guidos, C.J., Danska, J.S., Fathman, C.G., and Weissman, I.L. (1990). T cell receptor-mediated negative selection of autoreactive T lymphocyte precursors occurs after commitment to the CD4 or CD8 lineages. J. Exp. Med. *172*, 835–845.

Guidos, C.J., Williams, C.J., Wu, G.E., Paige, C.J., and Danska, J.S. (1995). Development of $CD4^+CD8^+$ thymocytes in RAG-deficient mice through a T cell receptor β chain-independent pathway. J. Exp. Med. *181*, 1187–1195.

Hashimoto, K., Sohn, S.J., Levin, S.D., Tada, T., Perlmutter, R.M., and Nakayama, T. (1996). Requirement for $p56^{lck}$ tyrosine kinase activation in T cell receptor-mediated thymic selection. J. Exp. Med. *184*, 931–943.

Itano, A., Salmon, P., Kioussis, D., Tolaini, M., Corbella, P., and Robey, E. (1996). The cytoplasmic domain of CD4 promotes the development of CD4 lineage T cells. J. Exp. Med. *183*, 731–741.

Jacobs, H., Vandeputte, D., Tolkamp, L., de Vries, E., Borst, J., and Berns, A. (1994). CD3 components at the surface of pro-T cells can mediate pre-T cell development *in vivo*. Eur. J. Immunol. *24*, 934–939.

Jameson, S.C., Hogquist, K.A., and Bevan, M.J. (1995). Positive selection of thymocytes. Annu. Rev. Immunol. *13*, 93–126.

Kawai, K., Kishihara, K., Molina, T.J., Wallace, V.A., Mak, T.W., and Ohashi, P.S. (1995). Impaired development of Vγ3 dendritic epidermal T cells in p56^{lck} protein tyrosine kinase-deficient and CD45 protein tyrosine phosphatase-deficient mice. J. Exp. Med. *181*, 345–349.

Kishi, H., Borgulya, P., Scott, B., Karjalainen, K., Traunecker, A., Kaufman, J., and Von Boehmer, H. (1991). Surface expression of the β T cell receptor (TCR) chain in the absence of other TCR or CD3 proteins on immature T cells. EMBO J. *10*, 93–100.

Levelt, C.N., and Eichmann, K. (1995). Receptors and signals in early thymic selection. Immunity *3*, 667–672.

Levelt, C.N., Mombaerts, P., Iglesias, A., Tonegawa, S., and Eichmann, K. (1993). Restoration of early thymocyte differentiation in T-cell receptor β-chain-deficient mutant mice by transmembrane signaling through CD3ε. Proc. Natl. Acad. Sci. USA *90*, 11401–11405.

Levelt, C.N., Mombaerts, P., Wang, B., Kohler, H., Tonegawa, S., Eichmann, K., and Terhorst, C. (1995). Regulation of thymocyte development through CD3: functional dissociation between p56^{lck} and CD3ζ in early thymic selection. Immunity *3*, 215–222.

Levin, S., Anderson, S., Forbush, K., and Perlmutter, R. (1993). A dominant-negative transgene defines a role for p56^{lck} in thymopoiesis. EMBO J. *12*, 1671–1680.

Ley, S.C., Marsh, M., Bebbington, C.R., Proudfoot, K., and Jordan, P. (1994). Distinct intracellular localization of Lck and Fyn protein tyrosine kinases in human T lymphocytes. J. Cell Biol. *125*, 639–649.

Liu, C.-P., Ueda, R., She, J., Sancho, J., Wang, B., Weddell, G., Loring, J., Kurahara, E., Dudley, E.C., Hayday, A., Terhorst, C., and Huang, M. (1993). Abnormal T cell development in CD3-ζ$^{-/-}$ mutant mice and identification of a novel T cell population in the intestine. EMBO J. *12*, 4863–4875.

Love, P.E., Shores, E.W., Johnson, M.D., Tremblay, M.L., Lee, E.J., Grinberg, A., Huang, S.P., Singer, A., and Westphal, H. (1993). T cell development in mice that lack the ζ chain of the T cell antigen receptor complex. Science *261*, 918–921.

Lowell, C.A., and Soriano, P. (1996). Knockouts of Src-family kinases: stiff bones, wimpy T cells, and bad memories. Genes Dev. *10*, 1845–1857.

Malissen, M., Gillet, A., Rocha, B., Trucy, J., Vivier, E., Boyer, C., Kontgen, F., Brun, N., Mazza, G., Spanopoulou, E., Guy-Grand, D., and Malissen, B. (1993). T cell development in mice lacking the CD3-ζ/η gene. EMBO J. *12*, 4347–4355.

Malissen, M., Gillet, A., Ardouin, L., Bouvier, G., Trucy, J., Ferrier, P., Vivier, E., and Malissen, B. (1995). Altered T cell development in mice with a targeted mutation of the CD3ε gene. EMBO J. *14*, 4641–4653.

McCarthy, S.A., Kruisbeek, A.M., Uppenkamp, I.K., Sharrow, S.O., and Singer, A. (1988). Engagement of the CD4 molecule influences cell surface expression of the T-cell receptor on thymocytes. Nature *336*, 76–79.

Molina, T.J., Kishihara, K., Siderovski, D.P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C.J., Hartmann, K.-U., Veillette, A., Davidson, D., and Mak, T.W. (1992). Profound block in thymocyte development in mice lacking p56^{lck}. Nature *357*, 161–164.

Mombaerts, P., Clarke, A., Rudnicki, M., Iacomini, J., Itohara, S., Lafaille, J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M., and Tonegawa, S. (1992a). Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. Nature *360*, 225–231.

Mombaerts, P., Iacomini, J., Johnson, R., Herrup, K., Tonegawa, S., and Papaioannou, V. (1992b). RAG-1-deficient mice have no mature B and T lymphocytes. Cell *68*, 869–877.

Mombaerts, P., Anderson, S.J., Perlmutter, R.M., Mak, T.W., and Tonegawa, S. (1994). An activated *lck* transgene promotes thymocyte development in RAG-1 mutant mice. Immunity *1*, 261–267.

Nakayama, T., Singer, A., Hsi, E.D., and Samelson, L.E. (1989). Intrathymic signaling in immature CD4$^+$8$^+$ thymocytes results in tyrosine phosphorylation of the T-cell receptor ζ chain. Nature *341*, 651–654.

Nakayama, T., June, C.H., Munitz, T.I., Sheard, M., McCarthy, S.A., Sharrow, S.O., Samelson, L.E., and Singer, A. (1990). Inhibition of T cell receptor expression and function in immature CD4$^+$ CD8$^+$ cells by CD4. Science *249*, 1558–1561.

Nakayama, T., Samelson, L.E., Nakayama, Y., Munits, T.I., Sheard, M., June, C.H., and Singer, A. (1991). Ligand-stimulated signaling events in immature CD4$^+$ CD8$^+$ thymocytes expressing competent T-cell receptor complexes. Proc. Natl. Acad. Sci. USA *88*, 9949–9953.

Negishi, I., Motoyama, N., Nakayama, K.-I., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A.C., and Loh, D.Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature *376*, 435–438.

Ohno, H., Aoe, T., Taki, S., Kitamura, D., Ishida, Y., Rajewsky, K., and Saito, T. (1993). Developmental and functional impairment of T cells in mice lacking CD3ζ chains. EMBO J. *12*, 4357–4366.

Olszowy, M.W., Leuchtmann, P.L., Veillette, A., and Shaw, A.S. (1995). Comparison of p56^{lck} and p59^{fyn} protein expression in thymocyte subsets, peripheral T cells, NK cells, and lymphoid cell lines. J. Immunol. *155*, 4236–4240.

Penninger, J., Kishihara, K., Molina, T., Wallace, V.A., Timms, E., Hedrick, S.M., and Mak, T.W. (1993). Requirement for tyrosine kinase p56^{lck} for thymic development of transgenic γδ T cells. Science *260*, 358–361.

Poussier, P., and Julius, M. (1995). T-cell development and selection in the intestinal epithelium. Semin. Immunol. *7*, 321–334.

Reynolds, P.J., Lesley, J., Trotter, J., Schulte, R., Hyman, R., and Sefton, B.M. (1990). Changes in the relative abundance of type I and type II *lck* mRNA transcripts suggest differential promoter usage during T-cell development. Mol. Cell. Biol. *10*, 4266–4270.

Seckinger, P., and Fougereau, M. (1994). Activation of *src* family kinases in human pre-B cells by IL-7. J. Immunol. *153*, 97–109.

Shinkai, Y., and Alt, F.W. (1994). CD3ε-mediated signals rescue the development of CD4$^+$CD8$^+$ thymocytes in RAG-2$^{-/-}$ mice in the absence of TCRβ chain expression. Int. Immunol. *6*, 995–1001.

Shinkai, Y., Rathbun, G., Lam, K.-P., Oltz, E., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A., and Alt, F. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855–867.

Shinkai, Y., Koyasu, S., Nakayama, K.-I., Murphy, K., Loh, D., Reinherz, E., and Alt, F. (1993). Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. Science *259*, 822–825.

Shores, E., Nakayama, T., Wiest, D., Takahama, Y., Sharrow, S., and Singer, A. (1993). Structurally distinct T cell receptor complexes on developmentally distinct T cell populations in severe combined immunodeficiency mice expressing a TCRβ transgene. J. Immunol. *150*, 1263–1275.

Stein, P.L., Lee, J.-M., Rich, S., and Soriano, P. (1992). pp59^{fyn} mutant mice display differential signaling in thymocytes and peripheral T cells. Cell *70*, 741–750.

Swan, K.A., Alberola-Ila, J., Gross, J.A., Appleby, M.W., Forbush, K.A., Thomas, J.F., and Perlmutter, R.M. (1995). Involvement of p21^{ras} distinguishes positive and negative selection in thymocytes. EMBO J. *14*, 276–285.

Tarakhovsky, A., Kanner, S.B., Hombach, J., Ledbetter, J.A., Müller, W., Killeen, N., and Rajewsky, K. (1995). A role for CD5 in TCR-mediated signal transduction and thymocyte selection. Science *269*, 535–537.

Teh, H.-S., Garvin, A.M., Forbush, K.A., Carlow, D.A., Davis, C.B., Littman, D.R., and Perlmutter, R.M. (1991). Participation of CD4 coreceptor molecules in T cell repertoire selection. Nature *349*, 241–243.

Turka, L.A., Linsley, P.A., Paine, R., Schieven, G.L., Thompson, C.B., and Ledbetter, J.A. (1991). Signal transduction via CD4, CD8, and CD28 in mature and immature thymocytes: implications for thymic selection. J. Immunol. *146*, 1428–1436.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L., and Tybulewicz, V.L.J. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature *378*, 298–302.

van Oers, N.S.C., Garvin, A.M., Davis, C.B., Forbush, K.A., Carlow,

D.A., Littman, D.R., Perlmutter, R.M., and Teh, H.-S. (1992). Disruption of CD8-dependent negative and positive selection of thymocytes is correlated with a decreased association between CD8 and the protein tyrosine kinase, p56^{lck}. Eur. J. Immunol. *22*, 735–743.

van Oers, N.S.C., von Boehmer, H., and Weiss, A. (1995). The pre-T cell receptor (TCR) complex is functionally coupled to the TCR-ζ subunit. J. Exp. Med. *182*, 1585–1590.

van Oers, N., Killeen, N., and Weiss, A. (1996). Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. J. Exp. Med. *183*, 1053–1062.

von Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E., and Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J. Exp. Med. *181*, 1519–1526.

Wallace, V.A., Kawai, K., Levelt, C.N., Kishihara, K., Molina, T., Timms, E., Pircher, H., Penninger, J., Ohashi, P., Eichmann, K., and Mak, T.W. (1995). T lymphocyte development in p56^{lck} deficient mice: allelic exclusion is incomplete but thymocyte development is not restored by TCRβ or TCRα transgenes. Eur. J. Immunol. *25*, 1312–1318.

Weiss, A., and Littman, D.R. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263–274.

Wells, F.B., Gahm, S.J., Hedrick, S.M., Bluestone, J.A., Dent, A., and Matis, L.A. (1991). Requirement for positive selection of $\gamma\delta$ receptor-bearing T cells. Science *253*, 903–905.

Wiest, D.L., Yuan, L., Jefferson, J., Benveniste, P., Tsokos, M., Klausner, R.D., Glimcher, L.H., Samelson, E., and Singer, A. (1993). Regulation of T cell receptor expression in immature CD4$^+$CD8$^+$ thymocytes by p56^{lck} tyrosine kinase: basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. J. Exp. Med. *178*, 1701–1712.

Wiest, D.L., Ashe, J.M., Abe, R., Bolen, J.B., and Singer, A. (1996). TCR activation of ZAP70 is impaired in CD4$^+$CD8$^+$ thymocytes as a consequence of intrathymic interactions that diminish available p56^{lck}. Immunity *4*, 495–504.

Wildin, R.S., Garvin, A.M., Pawar, S., Lewis, D.B., Abraham, K.M., Forbush, K.A., Ziegler, S.F., Allen, J.M., and Perlmutter, R.M. (1991). Developmental regulation of *lck* gene expression in T lymphocytes. J. Exp. Med. *173*, 383–393.

Wu, G., Danska, J.S., and Guidos, C.J. (1996). *Lck*-dependence of signaling pathways activated by γ-irradiation and CD3ϵ engagement of immature RAG-deficient thymocytes. Int. Immunol. *8*, 1159–1164.

Note Added in Proof

The data referred to as Groves et al., submitted, are now in press: Groves, T., Parsons, M., Miyamoto, N., and Guidos, C.J. (1997). TCR engagement of CD4$^+$CD8$^+$ thymocytes *in vitro* induces early aspects of positive selection, but not apoptosis. J. Immunol., in press.